ZOOLOGICAL SCIENCE 6: 423–426 (1989)

[COMMUNICATION]

Immunohistochemical Demonstration of Calcitonin Gene-Related Peptide in the Ultimobranchial Gland of Some Lower Vertebrates and in the Nervous Tissues of Some Invertebrates

Yuichi Sasayama, Kouhei Matsuda, Chitaru Oguro and Akira Kambegawa[1]

Department of Biology, Faculty of Science, Toyama University, Toyama 930, and [1]Department of Obstetrics and Gynecology, Faculty of Medicine, Teikyo University, Tokyo 173, Japan

ABSTRACT—Calcitonin gene-related peptide (CGRP) was detected immunohistochemically in the ultimobranchial gland of 2 species of cartilaginous fish and 3 species of frog, and in the nervous tissues of one species each of earthworm and sea-squirt.

INTRODUCTION

Calcitonin is a hormone secreted from the C-cells of the thyroid gland in mammals or from the ultimobranchial gland (UBG) in lower vertebrates. Recently, it was shown that the rat and human calcitonin genes encode a peptide other than calcitonin: calcitonin gene-related peptide (CGRP) [1–3]. This peptide has been identified immunochemically and immunohistochemically in the C-cells of the rat thyroid gland [4–6]. It is well known that the parenchymal cells of the UBG are embryologically homologous to the C-cells of the thyroid. However, it has not yet been determined whether the UBG is capable of producing CGRP. On the other hand, it has been reported that in some invertebrates, immunoreactive calcitonin exists in nervous tissues [7, 8]. This fact suggests that invertebrates may also possess the calcitonin gene that encodes CGRP. In the present study, therefore, an atempt was made to detect CGRP immunohistochemically in the UBG of some lower vertebrates and in nervous tissues of some invertebrates.

Accepted August 3, 1988
Received June 17, 1988

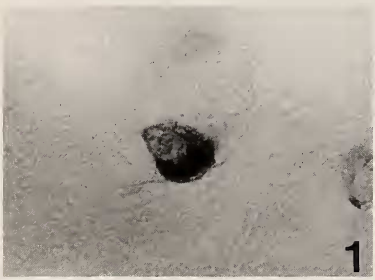
MATERIALS AND METHODS

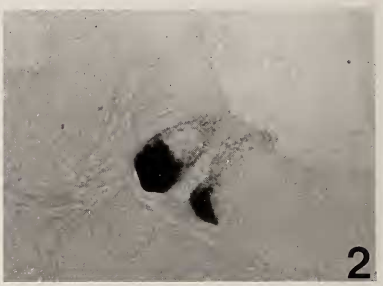
Twelve species of lower vertebrates examined. These were stingray (*Dasyatis akajei*) and guitarfish (*Rhinobatos schlegeli*) as representative cartilaginous fish, goldfish (*Carassius auratus*) as a representative bony fish, frogs (*Rana rugosa, R. nigromaculata,* and *R. catesbeiana*) and a newt (*Cynops pyrrhogaster*) as representative amphibians, snakes (*Rhabdophis tigrinus* and *Elaphe quadrivirgata*) and a lizard (*Takydromus tachydromoides*) as representative reptiles, and chicken (*Gallus domesticus*) as a representative bird. Furthermore, the earthworn (*Pheretima communissima*), an annelid, and the sea-squirt (*Styela plicata*), a protochordate were examined as representative invertebrates.

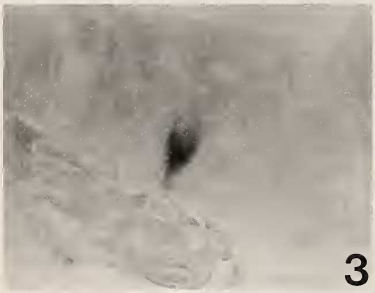
The UBG of lower vertebrates, the ventral nervous chain in the earthworm, and the neural complex of the sea-squirt were fixed in Bouin's solution (without acetic acid) for 5 hr, then dehydrated and embedded in paraffin. These were then sectioned at 6 μm according to routine procedures.

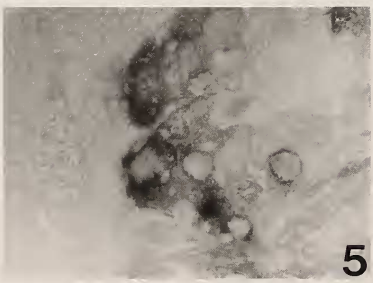
The double peroxidase-antiperoxidase (PAP) method was applied for the detection of CGRP.

Rabbit anti-rat CGRP antiserum (Amersham; diluted 1:900) was used as a primary antiserum at room temperature for 12 hr. Then, sections were incubated with anti-rabbit IgG porcine serum (1:20; Dako) and PAP (1:50; Dako), respectively for 30 min, in that order. Subsequently, anti-rabbit IgG and PAP were applied again, respectively for 30 min, in order to reinforce the reaction. Finally, the sections were treated with 0.05 M Tris-HCl buffer (pH 7.6) containing 3, 3'-









Figs. 1–5. CGRP immunoreactive cells in the UBG of some vertebrate species demonstrated by the PAP method. ×1120. Fig. 1. The sting-ray (*Dasyatis akajei*). Fig. 2. The guitar-fish (*Rhinobatos schlegeli*). Fig. 3. The frog (*Rana rugosa*). Fig. 4. The frog (*Rana nigromaculata*). Fig. 5. The frog (*Rana catesbeiana*).

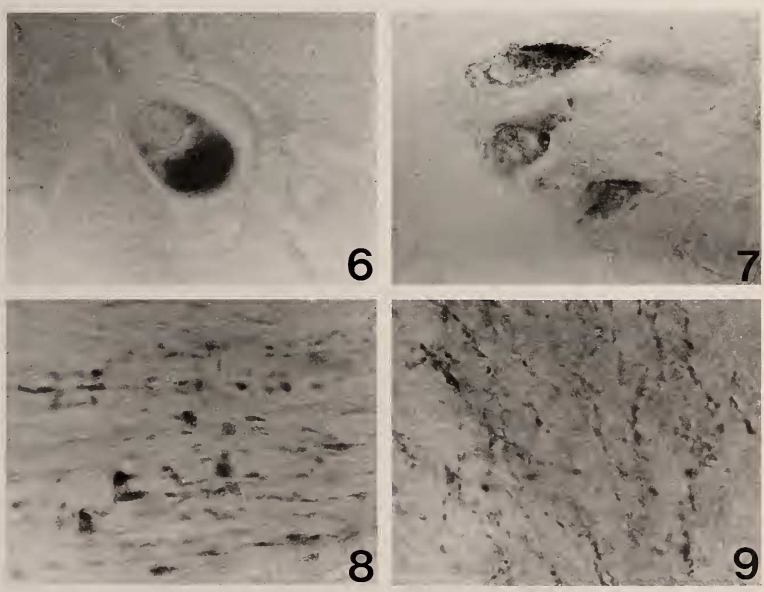
diaminobenzidine tetrahydrochloride and H_2O_2 solution.

The specificity was checked using primary antiserum inactivated by the addition of an excess amount of synthetic rat CGRP (7.7 μg/ml of the diluted antiserum). Furthermore, it was confirmed that the potency of the anti-CGRP antiserum was not damaged by incubation with synthetic salmon calcitonin (1.25 μg/ml). This result implies that anti-CGRP antiserum does not cross-react with calcitonin.

RESULTS AND DISCUSSION

CGRP immunoreactivity was detected in the UBG of the 2 species of cartilaginous fish and 3 species of frog, among the vertebrates examined. In other vertebrates examined in this study, no positive reaction was obtained in their UBG.

In the stingray and the guitar-fish, the UBG was composed of small follicles. The CGRP-immunoreactive cells were found dotted within the follicle wall (Figs. 1 and 2). In the latter species, the number of positive cells was rather smaller than in the former species. There was a tendency that in both species, the basal portion of positive cells was stained more strongly. In the frogs, the UBG was composed of a few follicles of various sizes. A few of the follicle cells located at the periphery of the gland reacted with the antiserum (Figs. 3–5). The number of positive cells in the UBG differed from species to species among these frogs, being more abundant in *R. rugosa* than in other 2 species. Also in the frog UBG, there was a



FIGS. 6–9. CGRP immunoreactivity in the nervous tissue of some invertebrate species demonstrated by the PAP method. Fig. 6. Cell in the ventral nervous chain of the earthworm (*Pheretima communissima*). Fig. 7. Cells in the cerebral ganglion of the sea-squirt (*Styela plicata*). Fig. 8. Immunoreactive axons in the earthworm (*Pheretima communissima*). Fig. 9. Immunoreactive axons in the sea-squirt (*Styela plicata*).

tendency for the basal portion of positive cells to be stained more strongly. These results show that the UBG in some species of lower vertebrates is capable of generating CGRP, as in the C-cells of higher vertebrates. In rats, it has been reported that CGRP is released at a rate similar to that of calcitonin [4]. Therefore, it seems that the amount of CGRP elaborated in the UBG of lower vertebrates is fairly small, even though it exhibits a positive reaction by the double PAP method. On the other hand, the physiological role CGRP present in C-cells has not yet been clarified. Also in the UBG of some lower vertebrates, its biological significance is unclear at the present time.

In the earthworm and the sea-squirt, CGRP immunoreactivity was detected in a few neurons of the nervous tissue. In the earthworm, the immunoreactive cells were found dotted within the peripheral region of the ventral nervous chain (Fig. 6). In the sea-squirt, the immunoreactive cells were found scattered in the peripheral portion of the cerebral ganglion (Fig. 7). It was noted that in these 2 species, many neuronal axons were also clearly stained (Figs. 8 and 9). Figure 10 shows schematically the location of the positive cells and axons. In rats, it has been reported that CGRP is also distributed in the brain in addition to the UBG. In the brain, this peptide is thought to play an important role as a neuromodulator [2]. Consequently, in the nervous tissues of some invertebrates, this putative function of CGRP may also apply.

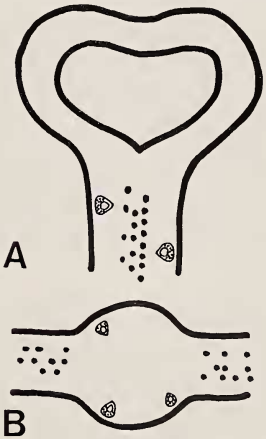


Fig. 10. Schematic drawings showing location of the CGRP immunoreactive cells and axons in the nervous tissues of the earthworm (A) and the sea-squirt (B).

The results obtained in the present study suggest a possibility that CGRP might have been encoded in the calcitonin gene at a fairly early stage of animal evolution. However, the problem of species specificity of the anti-CGRP autiserum used in the present study still remains. In order to generalize the significance of the present results, much more work needs to be done.

ACKNOWLEDGMENTS

We wish to express our sincere thanks to Dr. H. Michibata for kindly supplying the sea-squirts. The present study was supported in part by the Toyama Prefecture Centennial Foundation.

REFERENCES

1 Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. and Evans, R. M. (1982) Nature, **298**: 240–244.

2 Rosenfeld, M. G., Mermod, J-J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., Vale, W. W. and Evans, R. M. (1983) Nature, **304**: 129–135.

3 Morris, H. R., Panico, M., Etienne, T., Tippins, J., Girgis, S. I. and MacIntyre, I. (1984) Nature, **308**: 746–748.

4 Cooper, C. W., Borosky, S. A. and Peng, T-C. (1980) Proc. Soc. Exp. Biol. Med., **180**: 562–566.

5 Lee, Y., Kawai, Y., Shiosaka, S., Takami, K., Kiyama, H., Hillyard, C. J., Girgis, S., MacIntyre, I., Emson, P. C. and Tohyama, M. (1985) Brain Res., **330**: 194–196.

6 Lee, Y., Takami, K., Kawai, Y., Girgis, S. I., Hillyard, C. J., MacIntyre, I., Emson, P. C. and Tohyama, M. (1985) Neuroscience, **15**: 1227–1237.

7 Girgis, S. I., Galan, F., Arnett, T. R., Rogers, R. M., Bone, Q., Ravazzola, M. and MacIntyre, I. (1980) J. Endocrinol., **8**: 375–382.

8 Fritsch, H. A. R., Van Noorden, S. and Pearse, A. G. E. (1979) Cell Tiss. Res., **202**: 263–274.